Redox Biology 15 (2018) 347–362

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

Teaching the basics of reactive oxygen species and their relevance to cancer biology: Mitochondrial reactive oxygen species detection, redox signaling, and targeted therapies

Balaraman Kalyanaraman[a,b,c,*], Gang Cheng[a,b], Micael Hardy[d], Olivier Ouari[d], Brian Bennett[e], Jacek Zielonka[a,b,c]

[a] Department of Biophysics, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, United States
[b] Free Radical Research Center, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, United States
[c] Cancer Center, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, United States
[d] Aix Marseille Univ CNRS ICR UMR 7273, Marseille 13013, France
[e] Department of Physics, Marquette University, 540 North 15th Street, Milwaukee, WI 53233, United States

ARTICLE INFO

Keywords:
Reactive oxygen species
Triphenylphosphonium cation
Oxidative phosphorylation
Oxidative stress
Mitochondrial inhibition

ABSTRACT

Reactive oxygen species (ROS) have been implicated in tumorigenesis (tumor initiation, tumor progression, and metastasis). Of the many cellular sources of ROS generation, the mitochondria and the NADPH oxidase family of enzymes are possibly the most prevalent intracellular sources. In this article, we discuss the methodologies to detect mitochondria-derived superoxide and hydrogen peroxide using conventional probes as well as newly developed assays and probes, and the necessity of characterizing the diagnostic marker products with HPLC and LC-MS in order to rigorously identify the oxidizing species. The redox signaling roles of mitochondrial ROS, mitochondrial thiol peroxidases, and transcription factors in response to mitochondria-targeted drugs are highlighted. ROS generation and ROS detoxification in drug-resistant cancer cells and the relationship to metabolic reprogramming are discussed. Understanding the subtle role of ROS in redox signaling and in tumor proliferation, progression, and metastasis as well as the molecular and cellular mechanisms (*e.g.*, autophagy) could help in the development of combination therapies. The paradoxical aspects of antioxidants in cancer treatment are highlighted in relation to the ROS mechanisms in normal and cancer cells. Finally, the potential uses of newly synthesized exomarker probes for *in vivo* superoxide and hydrogen peroxide detection and the low-temperature electron paramagnetic resonance technique for monitoring oxidant production in tumor tissues are discussed.

1. Introduction

> "*Nonetheless, from a biological point of view, it is beginning to look as if ROS are neither cellular heroes nor villains—but instead something that occupies that always entertaining, captivating and fertile middle ground.*" *Holmstrom and Finkel (Nature Reviews)* [1]

Holmstrom and Finkel elucidated the dual nature of reactive oxygen species (ROS) that elicits both harmful and beneficial effects in cells and the state of the ROS in diseases including cancer [1]. Also, the authors emphasized the need to appreciate the differing chemistry of various ROS (*e.g.*, superoxide radical anion [$O_2^{\bullet-}$] and hydrogen peroxide [H_2O_2]) in redox-dependent pathways, highlighting the importance of developing methods to detect oxidants *in vivo*. In the present article, we address some of the gaps in our knowledge concerning ROS and redox signaling in cancer biology. Further, we discuss state-of-the-art assays and probes for detecting $O_2^{\bullet-}$, H_2O_2, and other oxidants in tumor cells in response to treatment with OXPHOS-targeting drugs, and their potential applications for the detection of mitochondria-derived ROS during tumorigenesis and metabolic reprogramming. The paradoxical role of ROS in tumor proliferation and tumor suppression [2] is discussed in the context of redox signaling mechanisms. Similarly, the paradoxical effects of antioxidants in tumorigenesis and tumor progression are discussed. Understanding the roles of mitochondrial ROS and redox signaling pathways in cancer biology may help in the discovery of relatively nontoxic and targeted therapies.

2. ROS: The most cited, most popular, yet most ambiguous term

The term "ROS" does not relate to a single species; rather, it covers a range of small molecule oxidizing, nitrosating, nitrating, halogenating,

* Corresponding author at: Department of Biophysics, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, United States
E-mail address: balarama@mcw.edu (B. Kalyanaraman).

https://doi.org/10.1016/j.redox.2017.12.012
Received 12 December 2017; Received in revised form 21 December 2017; Accepted 23 December 2017
Available online 26 December 2017

and thiol-reactive species, produced in biological systems. The use of ROS as an umbrella term for oxidants has been previously criticized because of its nonspecificity and ambiguity [3,4]. One of the authors of this article (BK) was also critical of using ROS as an umbrella term for all oxidants [3]. However, ROS as a term for small-molecule oxidants is now universally embraced and frequently used in novel biological settings by investigators in many areas of research, including cancer biology. Thus, it was decided that the same umbrella term, ROS, would be adapted for oxidants. That said, the lack of proper characterization of the structure of oxidants could seriously hamper our efforts to uncover new and novel oncogenic signaling pathways involved. In order to fully understand the signaling roles of ROS, it is essential to understand more about the nature and identity of the species, whether it is $O_2^{\bullet-}$, H_2O_2, lipid hydroperoxide, or an electrophile such as 4-hydroxynonenal derived from lipid oxidation. Proper identification of the structure of the ROS will also help us understand the mechanisms of action of drugs and drug resistance in cancer. In some ways, ROS levels and signaling are also modulated by other signaling molecules like nitric oxide ($^{\bullet}NO$) *via* a nearly diffusion-controlled reaction between $^{\bullet}NO$ and $O_2^{\bullet-}$ [5], generating a potent oxidizing and nitrating molecule, peroxynitrite ($ONOO^-$), also referred to as reactive nitrogen species (RNS). Although there is ample evidence for the occurrence of this type of mechanism and its biological relevance in cardiovascular and neurobiological systems [6,7], there is very little published data on the $^{\bullet}NO$ and $O_2^{\bullet-}$ interaction and its signaling ramifications in cancer biology. Many probes (fluorescent and chemiluminescent) have been previously employed to identify ROS, but there is still a lot of confusion in this field due to a lack of mechanistic rigor and the artifacts generated from reductive/oxidative activation of the probes themselves [8,9]. Most of these limitations have, however, been previously addressed [10,11]. Irrespective of the methodology used to detect ROS, it is clear that oxidants are involved, either as a major player or as a bystander, in the underlying biology. On the positive side, there now exist more specific probes and assays for selective identification of various ROS. Published data from independent laboratories are in agreement that identification of specific products formed from ROS interaction with fluorescent probes is crucial for determining the identity of ROS [12,13]. A reaction between $O_2^{\bullet-}$ and hydroethidine (HE) results in the formation of a very specific product, 2-hydroxyethidium (2-OH-E^+); this product is not formed from the reaction between HE and other biologically relevant oxidants such as H_2O_2, singlet oxygen, lipid hydroperoxides, peroxynitrite, HOCl, and $^{\bullet}NO_2$ [14,15]. This marker product derived from the $O_2^{\bullet-}$ and HE reaction (2-OH-E^+) can be unambiguously detected by rapid high-performance liquid chromatography (HPLC) and liquid chromatography–mass spectrometry (LC-MS) methods [16]. However, numerous publications using HE-derived fluorescence still posit that ethidium (E^+) is the product of the $O_2^{\bullet-}$ reaction with HE, whereas it has been clearly established by us and others that E^+ is not the product of the reaction between $O_2^{\bullet-}$ and HE (Fig. 1) [17,18]. Evidence also exists that the reaction chemistry between $O_2^{\bullet-}$ and other analogs of HE including Mito-SOX is similar to that of HE [19,20]. The lack of appreciation and the misconception of the chemistry and the mechanism of action of $O_2^{\bullet-}$ with HE, Mito-SOX (a mitochondria-targeted HE), and other HE analogs are responsible for the multitude of publications in biomedical research, including cancer, that suggest or conclude the intermediacy of $O_2^{\bullet-}$ formation [21,22].

Most assays to detect H_2O_2 are based on peroxidatic oxidation of probes such as Amplex Red in an extracellular milieu [23]. So far, very little information is available on chemical probes that react directly with H_2O_2 to form a diagnostic product. However, recently activity in this area has increased [24–26]. Boronate-based fluorescence probes react with H_2O_2 stoichiometrically (albeit very slowly, with the rate constant of 1–2 $M^{-1}\,s^{-1}$) to form fluorescent products [27,28]. Boronates also react with peroxynitrite nearly a million times faster than with H_2O_2, forming a major product (90%) that is the same as the product derived from the boronate/H_2O_2 reaction and a very characteristic and diagnostic minor product (5–10%) [27,29]. If the product that is highly diagnostic for peroxynitrite is not detected, it is likely that the major product is not formed from peroxynitrite (Fig. 2). Mitochondria-targeted boronates (*meta*-MitoB) were used to detect H_2O_2 *in vivo* [30,31]. We used an isomer, *ortho*-MitoB, to detect H_2O_2 because of its ability to distinguish between peroxynitrite and H_2O_2 [28,32,33]. Predicting the cellular response (activation of signaling pathways) to specific ROS requires a thorough understanding of its chemical properties in a biological setting.

3. Mitochondria, Nox, and ROS

Two major sources of ROS in cancer are mitochondria and nicotinamide adenine dinucleotide phosphate (NADPH) oxidases [34,35]. Mitochondrial respiratory chain complexes are generally thought to be responsible for generating ROS, $O_2^{\bullet-}$, and H_2O_2, in particular. Research in the early 1970s by Chance and collaborators provided the first evidence for mitochondrial generation of ROS [36]. Although $O_2^{\bullet-}$ formation in mitochondria was not convincingly demonstrated, Chance and coworkers demonstrated mitochondrial generation of H_2O_2 using a sensitive spectrophotometric method [36]. H_2O_2 was measured in the cytosolic extracts derived from mitochondria using the absorption changes that occur during the catalytic cycle of cytochrome *c* peroxidase and H_2O_2. This is a fundamentally significant discovery revealing an aberrant oxygen metabolism (albeit less than 1%) during mitochondrial respiration [37].

Complex III in the mitochondrial respiratory chain could form $O_2^{\bullet-}$ when mitochondria were treated with the inhibitor, antimycin. Mitochondrial complex I is another source of $O_2^{\bullet-}$ generation in the presence of rotenone that inhibits complex I [38]. Superoxide from complex I is also formed under conditions of a high proton motive force and reduced coenzyme Q pool (*i.e.*, a situation known as the reverse electron transport mechanism wherein electrons are driven back through complex I) [39].

That mitochondria also generate $O_2^{\bullet-}$ and H_2O_2 under *in vivo* conditions is supported by the existence of manganese superoxide dismutase (MnSOD) and other antioxidant enzymes (peroxidases and peroxiredoxins) in the mitochondrial matrix, and by the pathological consequences (*e.g.*, mitochondrial oxidative stress including DNA damage) resulting from their deficiency.

Nox enzymes are emerging as a promising target for anticancer drug development due to mounting evidence that suggests that NADPH/Nox-derived ROS inhibit tumor apoptosis and stimulate tumor proliferation [40,41]. Several Nox isoforms (*e.g.*, Nox2 and Nox4) have been proposed as potential therapeutic targets in the treatment of cancer and other diseases [42]. Unlike other redox enzymes for which ROS generation is an “accidental” byproduct of their primary catalytic function, the only known function of Nox enzymes (Nox1-5, Duox1-2) is generation of ROS (*e.g.*, $O_2^{\bullet-}$ and H_2O_2) [43]. Nox2 forms both $O_2^{\bullet-}$ and H_2O_2 (*via* dismutation of $O_2^{\bullet-}$); however, published reports suggest that Nox4 primarily generates H_2O_2 (90%) [44,45]. A major impediment to advancing Nox research in cancer biology is the lack of availability of selective inhibitors of Nox isoforms [46]. This, in turn, had been due to the lack of assays selective for $O_2^{\bullet-}$ and H_2O_2 using specific probes, but this hurdle has been largely overcome with recent discoveries of new probes and sensitive assays for detection of ROS and RNS [47].

Oncogenic KRAS was reported to promote ROS/RNS generation by increasing the expression and activity of Nox enzymes at the tumor cell membrane [48]. However, it is likely that Nox activity is modulated by changes in mitochondrial bioenergetics. Although there are reports in the vascular biology literature of potential “cross-talk” between mitochondrial ROS and Nox activation [49], there is no information, to our knowledge, on the modulatory role of mitochondrial metabolism on Nox/ROS metabolism and oxidative signaling in cancer biology. Although, this particular aspect is outside the scope of the present review, understanding how modifications of cancer cell bioenergetics and